The homeobox gene *NKX3.2* is a target of left–right signalling and is expressed on opposite sides in chick and mouse embryos

André Schneider*, Tomek Mijalski*, Thomas Schlange*, Weilie Dai†, Paul Overbeek†, Hans-Henning Arnold* and Thomas Brand*

Vertebrate internal organs display invariant left–right (L–R) asymmetry. A signalling cascade that sets up L–R asymmetry has recently been identified (reviewed in [1]). On the right side of Hensen's node, activin represses *Sonic hedgehog* (*Shh*) expression and induces expression of the genes for the activin receptor (ActRIIa) and fibroblast growth factor-8 (FGF8) [2,3]. On the left side, *Shh* induces *nodal* expression in lateral plate mesoderm (LPM); *nodal* in turn upregulates left-sided expression of the *bicoid*-like homeobox gene *Pitx2* [4–6]. Here, we found that the homeobox gene *NKX3.2* is asymmetrically expressed in the anterior left LPM and in head mesoderm in the chick embryo. Misexpression of the normally left-sided signals Nodal, Lefty2 and Shh on the right side, or ectopic application of retinoic acid (RA), resulted in upregulation of *NKX3.2* contralateral to its normal expression in left LPM. Ectopic application of FGF8 on the left side blocked *NKX3.2* expression, whereas the FGF receptor-1 (FGFR-1) antagonist SU5402, implanted on the right side, resulted in bilateral *NKX3.2* expression in the LPM, suggesting that FGF8 is an important negative determinant of asymmetric *NKX3.2* expression. *NKX3.2* expression was also found to be asymmetric in the mouse LPM but, unlike in the chick, it was expressed in the right LPM. In the *inversion of embryonic turning* (*inv*) mouse mutant, which has aberrant L–R development, *NKX3.2* was expressed predominantly on the left side. Thus, *NKX3.2* transcripts accumulate on opposite sides of mouse and chick embryos although, in both the mouse and chick, *NKX3.2* expression is controlled by the L–R signalling pathways.

Addresses: *Department of Cell and Molecular Biology, Technical University of Braunschweig, Germany. †Department of Cell Biology, Baylor College of Medicine, Houston, USA.

Correspondence: Thomas Brand
E-mail: t.brand@tu-bs.de

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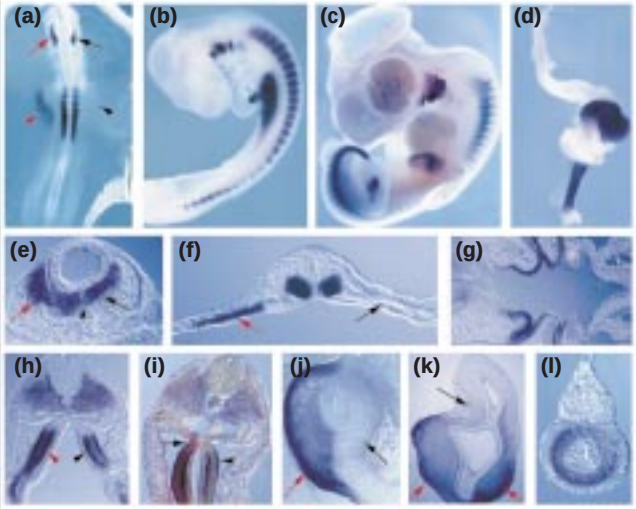
Results and discussion

The NK family of homeobox genes is involved in cellular differentiation and organogenesis [7]. We identified the chicken homologue of mouse *BapX1* (also known as *NKX3.2*) [8], from here on referred to as chicken *NKX3.2*. Transcripts for *NKX3.2* were first detected by whole-mount *in situ* hybridization at stage 9 of embryonic development in the somites. During early stages of somitogenesis (stages 9–10), expression appeared transiently stronger on the left side (data not shown). Beginning at stage 10 (12 somites), *NKX3.2* was expressed in the left but not the right LPM in a domain spanning approximately the first four somites (data not shown). At stage 11 (15 somites), *NKX3.2* was expressed in the left LPM along the first eight somites (Figure 1a,f). With further development, *NKX3.2* expression shifted posteriorly and, at stage 18 (30 somites), expression was confined to a domain in the LPM between somites 5 and 13 (Figure 1b). *NKX3.2* was also expressed in head mesoderm with consistently higher levels on the left side (Figure 1a,e). Beginning at stage 13, *NKX3.2* transcripts also accumulated in the right LPM but expression levels remained lower than on the left side and extended less in the anteroposterior (AP) direction (Figure 1h).

Double-colour *in situ* hybridization revealed that *Pitx2* and *NKX3.2* expression domains overlapped; *Pitx2* was, however, expressed in the entire left LPM [4–6] and the expression domain was also wider in the dorsoventral direction. In contrast to *NKX3.2*, *Pitx2* was completely absent from right LPM (Figure 1i). The differential expression of *NKX3.2* and *Pitx2* along the AP axis might be important for diversifying L–R specification. At later stages of development, *Pitx2* and *NKX3.2* expression domains were non-overlapping in the digestive tract (data not shown). At stage 18, when the gut tube is fused, *NKX3.2* expression was found in the stomach and epithelium of the pharyngeal arches (Figure 1g). From stage 24 onwards, *NKX3.2* was also expressed in forelimbs and hindlimbs (Figure 1c). In the stomach, *NKX3.2* was predominantly expressed on the left side and, at embryonic day 7, it was precisely confined to mesenchyme and forming muscle layers in gizzard and absent from the pylorus (Figure 1d,j,k). In the hindgut, we found almost perfectly symmetric *NKX3.2* expression in the mesenchymal layer surrounding the gut epithelium (Figure 1l).

To determine whether the asymmetric expression of *NKX3.2* in the LPM was under the control of the L–R signalling cascade, we implanted aggregates of embryonic fibroblasts expressing Shh, Nodal or Lefty2 from a retrovirus, or beads loaded with RA, into chick embryos cultured according to New [9] (Figure 2). In these experiments,

Figure 1



NKX3.2 is expressed asymmetrically in the LPM, head mesoderm and stomach. (a) Dorsal view of stage 11 embryo showing *NKX3.2* expression in left LPM (red arrowhead) but not in right LPM (black arrowhead), and asymmetric expression in head mesoderm (arrows). (b,c) Left lateral views of embryos showing *NKX3.2* expression at (b) stage 18 in pharyngeal arches, stomach and somites; and (c) stage 24 in pharyngeal arches, stomach, sclerotome, forelimbs and hindlimbs. (d) Digestive tract of a day 7 embryo (left lateral view) showing segmented *NKX3.2* expression in gizzard and hindgut. (e,f) Transverse sections through the embryo in (a). (e) *NKX3.2* expression in head mesoderm was stronger on the left side (red arrow) than on the right (black arrow); the arrowhead indicates the notochord. (f) *NKX3.2* was expressed in left LPM (red arrow) but not in right LPM (black arrow); expression in the somites was bilateral. (g,h) Transverse sections at the cranial level through the embryo in (b). *NKX3.2* was expressed symmetrically in (g) epithelium of the pharyngeal arches and in (h) sclerotome, but asymmetrically in splanchnic mesoderm (red arrowhead, left side; black arrowhead, right side). (i) Transverse section through stage 18 embryo subjected to double-colour whole-mount *in situ* hybridization with probes for *Pitx2* (red staining) and *NKX3.2* (blue staining). Expression of both genes overlapped in left splanchnic mesoderm but not in the dorsal-most splanchnic mesoderm (black arrow), and in right splanchnic mesoderm (black arrowhead). (j) Transverse section through the stomach of the embryo in (c) showing *NKX3.2* expression only in the left half of the stomach. (k,l) Transverse sections through the (k) gizzard and (l) hindgut of the digestive tract shown in (d). (k) *NKX3.2* was strongly expressed in mesenchyme and muscle layer of the outer curvature (derivative of the left splanchnic mesoderm; red arrows) and weakly in the inner curvature (derivative of the right splanchnic mesoderm; black arrow). (l) *NKX3.2* was almost symmetrically expressed in mesenchyme of the hindgut.

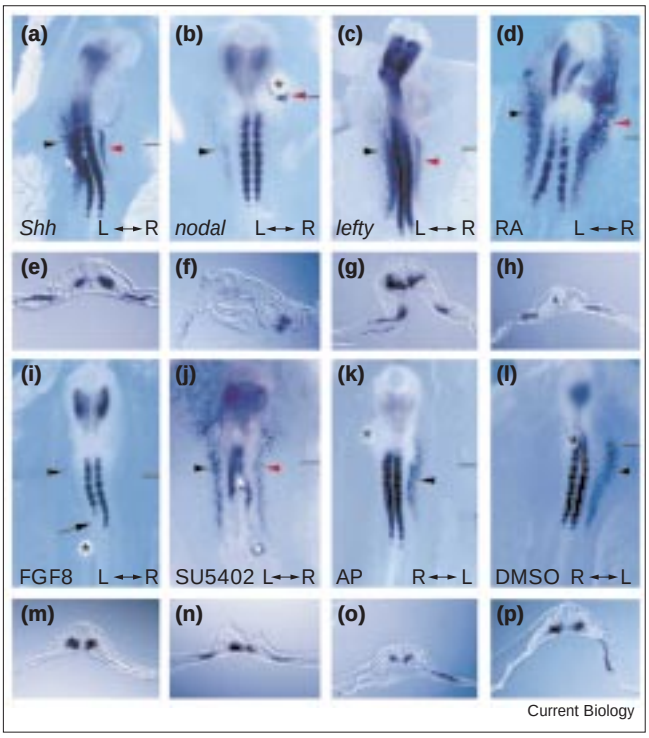
Lefty2 was the most effective inducer and strongly induced bilateral *NKX3.2* expression in 18 of 31 implanted embryos. In optimal cases, ectopic *NKX3.2* expression was observed almost along the entire right LPM (Figure 2c). Shh induced bilateral *NKX3.2* expression in 12 out of 43 manipulated embryos, and RA in 9 out of 38 cases (Figures 2a,d). The least effective inducer was Nodal; *NKX3.2* expression was induced only in cells surrounding the implant in 5 out of 36 cases (Figure 2b). The differential response of *NKX3.2* to these signals might simply reflect differences in efficiency of viral infections or expression levels of the secreted proteins. Control embryos implanted with cells expressing alkaline phosphatase (AP), or beads loaded with dimethylsulfoxide (DMSO), occasionally showed inverse heart looping (4 out of 43 embryos) but never right-sided *NKX3.2* expression.

We next investigated the response of *NKX3.2* to FGF8, which might function as negative regulator of left-side signals [2]. Significantly, implantation of FGF8-soaked beads on the left side eliminated or greatly diminished *NKX3.2* expression in the left LPM, somites and head mesoderm in 12 out of 16 embryos (Figure 2i,m). Moreover, FGF8-loaded beads implanted on the right side blocked *NKX3.2* expression in the somitic mesoderm in 3 out of 4 embryos (data not shown). To test whether endogenous FGF signalling actually prevented the expression of *NKX3.2* on the right side, the FGFR-1 inhibitor SU5402 [10] was implanted; this resulted in bilateral expression of *NKX3.2* in 3 out of 5 embryos (Figure 2j,n), confirming that FGF signalling is essential to establish the asymmetric expression of *NKX3.2* in chick embryos.

We next analyzed whether the *BapX1*/*NKX3.2* gene was also expressed asymmetrically in mouse. Consistent with previous reports, *BapX1*/*NKX3.2* was first expressed in somites, just like its avian orthologue (Figure 3a,b) [8]. Mouse *BapX1*/*NKX3.2* was also asymmetrically expressed in the LPM of embryos at 8.5 days *post coitum* (dpc) but, in contrast to the chick embryo, *BapX1*/*NKX3.2* transcripts were predominantly expressed in right LPM and only weakly in the left LPM (Figure 3a,e). At 8.75 dpc, expression became also visible in the left LPM, but remained clearly weaker and in a smaller domain. At 9.5 dpc, *NKX3.2* transcripts were found in the stomach and sclerotome (Figure 3b). The stomach mesoderm displayed slightly stronger expression of *BapX1*/*NKX3.2* on the right side (Figure 3f). *BapX1*/*NKX3.2* expression in the mouse LPM on the opposite side to that in the chick prompted us to investigate whether the gene was also under the control of L–R signalling in the mouse embryo. We used the *inv* mouse mutant which displays complete *situs inversus* resulting from the deletion of the ankyrin-repeat-containing protein *inversin* [11,12]. In contrast to wild-type embryos, homozygous *inv* mutant embryos (3 out of 3 embryos) showed highly asymmetric expression of *BapX1*/*NKX3.2* in the left LPM. In summary, *NKX3.2* is expressed on opposite sides in mouse and chick embryos, but our data suggest that the gene is under the control of the L–R signalling pathway in both species. Therefore, important differences exist in the way the L–R axis is established in these two vertebrates.

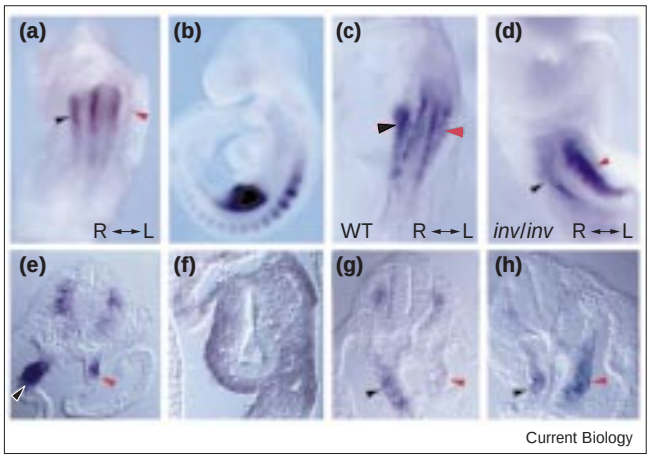
We have shown here that asymmetric expression of *NKX3.2* in the left LPM of the chick embryo is the result of left-sided induction by known signalling molecules

Figure 2



NKX3.2 expression in the LPM is regulated by L–R signalling molecules. **(a–d,i,j)** Dorsal and **(k,l)** ventral views of chick embryos, showing *NKX3.2* expression detected by whole-mount *in situ* hybridization. (a–d) Embryos were implanted on the right side either with (a–c) embryonic fibroblasts infected with retroviruses expressing (a) *Shh*, (b) *nodal*, (c) *lefty2*, or (d) RA-loaded beads. Arrowheads, ectopic (red) and endogenous (black) *NKX3.2* expression in the LPM. **(e–h)** The corresponding transverse sections at the implant level of the embryos shown in (a–d). (i) Endogenous *NKX3.2* expression in the LPM (arrowhead) and somites (arrow) was inhibited by implanting beads that were soaked in FGF8 on the left side. (j) Embryo implanted with beads loaded with the FGFR-1 inhibitor SU5402 on the right side exhibited bilateral *NKX3.2* expression in the LPM. Control embryos implanted with (k) embryonic fibroblasts infected with a retrovirus expressing alkaline phosphatase (AP), or (l) beads loaded with dimethylsulfoxide (DMSO), exhibited normal expression of *NKX3.2* only in left LPM. **(m–p)** The corresponding transverse sections through the embryos shown in (i–l). Asterisks, location of beads or cell implants (not all have been indicated); bars, approximate plane of sections; L↔R, the left–right orientation of the embryo.

Figure 3

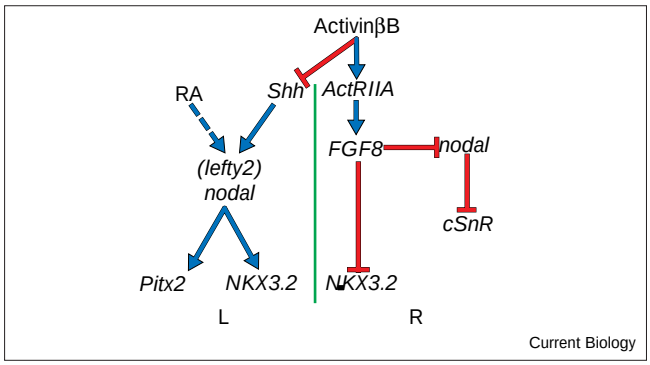


In mice, *NKX3.2* is expressed in the right LPM and reversed in the *inv* mutant. **(a,c,d)** Ventral and **(b)** left side views of mouse embryos, showing *NKX3.2* expression detected by whole-mount *in situ* hybridization. (a) At 8.0 dpc, *NKX3.2* was expressed symmetrically in somites and asymmetrically in the LPM, predominantly on the right side. (b) At 9.5 dpc, *NKX3.2* was strongly expressed in stomach and somites. (c) Wild-type (WT) embryo and (d) *inv/inv* littermate. In *inv/inv* embryos (d), *NKX3.2* was expressed symmetrically in somites and predominantly in the left LPM in contrast to the predominantly right-sided expression in the wild-type littermate (c). **(e–h)** The corresponding transverse sections of the embryos shown in (a–d). Red arrowhead, *NKX3.2* expression in the left LPM; black arrowhead, *NKX3.2* expression in the right LPM; R↔L, the right–left orientation of the embryos.

including Shh, Nodal and Lefty2, and also by RA, which in addition to Shh, is probably required for the expression of the *nodal* and *lefty* genes [13] (Figure 4). Currently only *lefty1* has been identified in chicken (J.C. Izpisúa-Belmonte, personal communication). Both *lefty1* and *lefty2* genes, however, are present in zebrafish [14] and mouse [15] making it very likely that they are also present in the chicken genome. As expression of *NKX3.2* and *Pitx2* overlapped only partially in the left LPM, it is likely that both genes are independently activated by the L–R signalling cascade. We also observed right-sided repression of *NKX3.2* by FGF8, which may be a major determinant of the one-sided expression pattern in the chick embryo. Our experiments do not allow us to distinguish formally whether FGF8 represses *NKX3.2* transcription directly, possibly through the induction of the transcriptional repressor *cSnR* (chick *snail-related*) [16], or prevents synthesis of the left-sided signals, such as Nodal on the right side, or both [17].

Birds and mammals differ in several aspects of L–R symmetric morphogenesis. In avians the stomach becomes asymmetric by differential growth, whereas in mammals organ rotation appears to be an important event [18,19]. The *aorta descendens* is formed in the mouse by the left branch of the fourth branchial arch artery whereas it is the right branch in birds [20]. The observed expression of *NKX3.2* on opposite sides in mouse and chicken may reflect some of these developmental differences, particularly in stomach organogenesis. Moreover, the reversal of sides raises significant questions concerning the conservation of L–R patterning during evolution. Interestingly, the *Xenopus* orthologue *Xbap* has also been found to be asymmetrically expressed on the left side of the anterior gut mesoderm [21]. In contrast to the mouse and chicken orthologues, however, *Xbap* is initially expressed bilaterally and, only later in development, does expression become L–R asymmetric.

Figure 4



Model of the molecular pathways involved in the generation of L–R asymmetry in *NKX3.2* expression in the chick. RA induces *NKX3.2* expression most likely through *lefty2* or *nodal*; this relationship has, however, only been established in the mouse embryo [15]. *Lefty2* has been indicated in brackets. *FGF8* on the right side might block *NKX3.2* expression directly, or via the blockade of the left-sided signals such as *nodal* leading to the right-sided expression of *cSnR*.

It is interesting to note that *nodal* and *Pitx2*, the downstream components of the L–R signalling cascade, are expressed in the left LPM in fish, frog, chicken and mouse [4–6,19], but the upstream signalling molecules, such as Shh and FGF8, have been shown to be asymmetrically expressed in the chicken but not in *Xenopus* or mouse [22–24]. Nevertheless, ectopic expression of *Shh* in *Xenopus* results in ectopic induction of *nodal* [25]. Moreover, *Shh* null mutant mice display laterality defects such as left isomerism of the lung and bilateral *nodal*, *lefty2* and *Pitx2* expression [26]. Loss of *FGF8* function in mice also causes a dramatic double-right-sided phenotype, suggesting that *FGF8* in the mouse controls left-sided gene expression [27]. Thus, although most molecules involved in L–R asymmetry appear to be conserved, they may have different roles in different species.

Supplementary material

Supplementary material including methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

The homeobox gene *NKX3.2* is a target of left–right signalling and is expressed on opposite sides in chick and mouse embryos

André Schneider, Tomek Mijalski, Thomas Schlange, Weilie Dai, Paul Overbeek, Hans-Henning Arnold and Thomas Brand

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Supplementary materials and methods

Cloning of NKX3.2 *cDNA and* in situ *hybridisation*

The chick embryonic stage 12–15 cDNA library was screened using mouse *NKX3.2* cDNA as a probe and the chicken homologue of mouse BapX1 (also known as NKX3.2) was identified [S1]. Strong sequence conservation within the homeobox, the NK2 specific domain (NK2SD) and the tinman (TN) domain and the entire carboxy-terminal sequence suggested that chicken *NKX3.2* was orthologous to mouse *BapX1/NKX3.2*, *Xenopus Xbap* [S2] and the urodele *Pleurodeles waltl PwNkx-3.2* [S3]. The sequence was deposited in GenBank (accession number AF138905). Whole-mount *in situ* hybridisation and sectioning of chick embryos (staged according to Hamburger and Hamilton [S4]) were performed as described [S5]. Antisense RNA probes for chicken *Pitx2* and chicken and mouse *NKX3.2* were generated from full-length cDNA clones [S6,S7]. Double-colour whole-mount *in situ* hybridisation was performed as described [S8].

Generation of cell aggregates and bead preparation

Chick embryonic fibroblasts were transfected with RCAS-BP(A) constructs encoding Shh [S9], mature chick Nodal fused to the proregion of the bone morphogenetic protein-4 (BMP4) [S10], mouse Lefty2 [S11] or alkaline phosphatase [S12] and aggregated as described previously [S13]. AG1-X2 ion-exchange resin (BioRad) beads (mesh size 200–400) were incubated for 20 min with 50 µg/µl all-*trans* retinoic acid (SIGMA) [S14], or with 2 mM SU5402 (Calbiochem-Novabiochem) [S15], both solubilised in DMSO. Control beads were incubated in DMSO only. Heparin acryl beads (SIGMA) were incubated for 30 min with 100 ng/µl mouse FGF8b (R&D Systems) [S16]. For implantation, beads were washed three times for 30 sec each in Pannett–Compton buffer.

Embryo manipulation

White Leghorn eggs (SPAFAS, Charles River, Extertal) were incubated until stage 4. The embryos were set up as New cultures [S17] and implanted with aggregates of Shh-producing fibroblast, or beads loaded with RA or SU5402, on the right side of Hensen's node. FGF8 beads were implanted on the left side at the same developmental stage. Aggregates of fibroblasts secreting Nodal or Lefty2 were implanted at stage 7 in the right LPM at the level of the first somite. Cultured embryos were fixed in buffered 4% paraformaldehyde at stage 11 and subjected to whole-mount *in situ* hybridisation as described [S5].

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